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Effects of raw meat and process procedure on N$^{\varepsilon}$-carboxymethyl-lysine and N$^{\varepsilon}$-carboxyethyl-lysine formation in meat products

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Abstract This work aimed to investigate the effects of stored raw meat and process procedures on N$^{\varepsilon}$-carboxymethyl-lysine (CML) and N$^{\varepsilon}$-carboxyethyl-lysine (CEL) generation in meat products. Meat products of raw pork were sterilized and pasteurized at different storage times (0-4 months) and the CML/CEL contents were determined. The results showed that the extent of lipid and protein oxidation of raw pork increased with increasing storage time. A linear correlation was found between thiobarbituric acid-reactive substances value/carbonyl content and CML/CEL in sterilized meat products, indicating that stored raw pork could promote CML/CEL formation under high temperature processing. Furthermore, mild heating temperatures seemed to favor CML formation, while high temperature could accelerate CEL generation. Therefore, formation kinetics of CML and CEL might be different for different process procedures. These results suggested that stored raw meat and processing temperature could significantly affect CML and CEL generation in meat products.

Keywords: advanced glycation end products, lipid oxidation, meat products, N$^{\varepsilon}$-carboxymethyl-lysine, N$^{\varepsilon}$-carboxyethyl-lysine

Introduction

Advanced glycation end products (AGEs) are a series of complex compounds produced through nonenzymatic reactions between reducing sugars and amino compounds during food processing and storage (1). These compounds are believed to be associated with many chronic diseases. It has been found that N$^{\varepsilon}$-carboxymethyl-lysine (CML) and N$^{\varepsilon}$-carboxyethyl-lysine (CEL), as typical AGE biomarkers, might cause kidney and liver dysfuntion for individuals (2) and contribute to the development of diabetes (3) and Alzheimer's disease (4). Daily diet is an important source of endogenous CML and CEL pools. Therefore, the study of CML and CEL is of current interest, and many studies have been conducted to elucidate the influencing factors of CML and CEL formation in foods (5,6).

Meat products, due to the high protein/fat content, easily generate CML and CEL during processing and storage. In meat products, CML and CEL can be formed not only by the Maillard reaction pathways but also by the lipid oxidation pathways (7). The contents of CML and CEL in meat products depend on several factors such as cooking methods, storage conditions, and raw material type (8,9). For example, Goldberg *et al.* (10) reported that high-fat meat products (such as pork, beef, and chicken) have higher CML content than low-fat meat products or carbohydrate-rich foods. Chen and Smith (6) suggested that fried or broiled meat products could produce high content of CML. Hull *et al.* (8) also reported that CML content in meat and fish products varied from 0.76 to 286.31 mg/kg protein, which indicated that CML generation was significantly affected by the types of raw meat. Furthermore, there have been some reports describing generation kinetics of CML in the model system, such as the casein-glucose-fatty acid model system (11), saccharide-lysine model system (12), and lysine-oil-glucose model system (13). Although the generation mechanism of CML in the model food system has made rapid progress, the food system has numerous components that may affect AGEs formation, and the simple model systems can not fully reveal what actually happens regarding CML and CEL formation in real food systems. These studies paid more attention to the effects of cooking methods, types of raw meat, and heating time on CML formation in meat products or model systems. However, information regarding the effects of oxidation of raw meat and heat sterilization methods on CML and CEL generation is still limited.

Generally, the meat products in the market are mainly made from frozen raw meat and processed at high temperature. During storage and processing, lipid and protein oxidation will inevitably occur, which may affect the generation of CML and CEL in meat products (14,15). Therefore, the objectives of the present study were to investigate the effects of oxidation of raw meat and heat sterilization

methods on the generation of CML and CEL, thereby providing useful information for reducing CML and CEL formation in meat products.

Materials and Methods

Materials Sodium borate, sodium borohydride, thiobarbituric acid (TBA), trichloroacetic acid (TCA), petroleum ether, and n-hexane were purchased from Sinopharm Chemical Reagent (Shanghai, China). CML, CEL, d_4-CML, and d_4-CEL were supplied by Santa Cruz Biotechnology (Dallas, TX, USA). Nonafluoropentanoic acid (NFPA) and HPLC grade methanol and acetonitrile were obtained from JK Chemical (Beijing, China). Raw pork and commercial meat products were purchased from local supermarkets (Wuxi, China).

Preparation of sterilized and pasteurized meat products Sterilized and pasteurized meat products were prepared and sterilized with the methods of Cheon e*t al.* (16) and Desmond and Kenny (17) with some modifications, as shown in Fig. 1. Briefly, lean pork was trimmed, cleaned thoroughly, and cut into small pieces (about 1 cm^3). The lean pork was cured with sodium chloride (2.0%, w/w) at 4°C for 48 h and packaged with a retort pouch by a vacuum sealer. For sterilization, meat products were sterilized at 121°C for 30 min and stored at 4°C for further analysis. For pasteurization, meat products were pasteurized at 85°C for 4 h and stored at 4°C for further analysis.

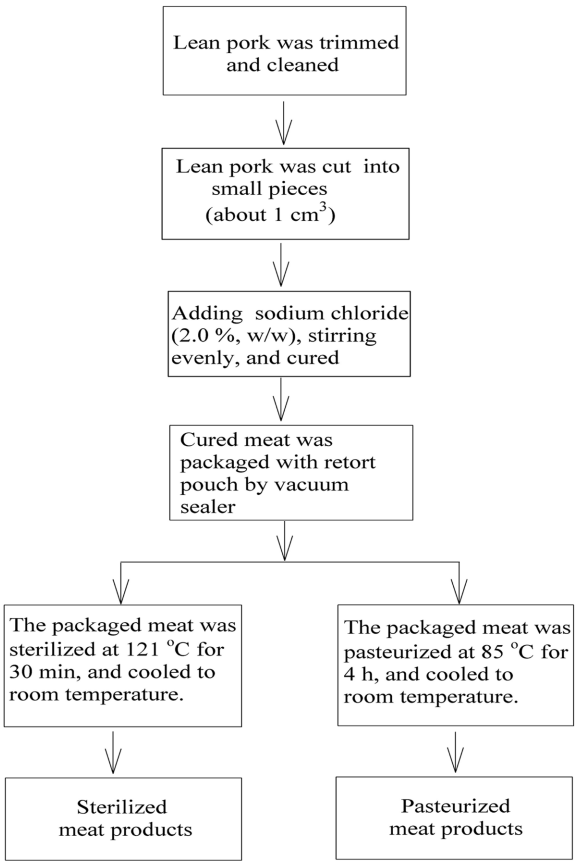

Fig. 1. Processing procedure of sterilized and pasteurized meat products.

Protein and moisture content determination The Kjeldahl procedure was employed to estimate the protein content according to the AOAC Official Method 928.08, Protein content (18), and moisture content was determined according to the AOAC Official Method 950.46, Moisture content in meat (19).

Determination of lipid oxidation in raw pork Raw pork was stored at 18°C for 0, 1, 2, 3, and 4 months, and the extent of lipid oxidation was determined using thiobarbituric acid-reactive substances (TBARs) according to Wang and Xiong (20) with slight modifications. Briefly, stored raw pork was lyophilized and powdered with a micro Waring blender. Then, a 0.5 g chopped meat sample was placed in a 25 mL screw cap test tube. Three drops of antioxidant solution (BHA) and 3 mL of TBA solution were added, respectively. Then, the sample was vortex mixed for 60 s to disperse the sample, and an aliquot of 17 mL TCA-HCl solution was added to the mixture. The reaction was initiated by heating at 95°C for 60 min. After being cooled to room temperature, a 5 mL aliquot of the supernatant was mixed with 5 mL chloroform, vortex mixed for 60 s, and then centrifugated for 10 min at 1,680× *g*. Finally, 4.5 mL of the upper phase was aspirated into another test tube and thoroughly mixed with 4.5 mL of petroleum ether. The lower phase was taken, and its absorbance read at 532 nm against a reagent blank. The blank was prepared with 1 mL of deionized distilled water. The result was expressed as mg malonaldehyde (MDA)/kg sample. The TBARs value was calculated with the following equation:

$$\text{TBARs (mg/kg sample)} = \frac{A_{532}}{\text{Ws}} \times 9.48$$

where A_{532} and Ws represent the absorbance at 532 nm and weight (g) of sample, respectively, and "9.48" is a constant.

Carbonyl content in raw pork Different storage times of frozen raw pork were tempered at 4°C for 4 h and then used for extraction of myofibrillar protein (MFP) using the method of Park and Xiong (21). Briefly, muscle samples were chopped into small pieces, and the isolation buffer (0.1 M NaCl, 2 mM $MgCl_2$, 10 mM sodium phosphate, and 1 mM EGTA, pH 7.0) was added. In the last washing step, the pH of MFP suspension at 0.1 M NaCl was adjusted to 6.25 using 1.0 M HCl before centrifugation. The protein concentration was determined by the method of Biuret (22). Sample carbonyl content was measured using the 2,4-dinitrophenylhydrazine (DNPH) colorimetric method of Liu *et al*. (23) with some modifications. Briefly, the DNPH-reacted MFP samples were precipitated using 20% TCA and then recovered by centrifugation. The precipitated MFP pellets were washed three times using ethanol-ethyl acetate solution (1:1, v/v) and then dissolved in 6 M guanidine hydrochloride. The absorbance at 370 nm was recorded for carbonyl content and that at 280 nm was read for

protein concentration. The carbonyl content was calculated as follows:

$$\text{Carbonyls (nmol/mg protein)} = \frac{A_{370}}{\varepsilon} \times \frac{10^6}{C}$$

where A_{370} and C represent the absorbance at 370 nm and protein concentration (mg/mL), respectively. "ε" is a molar extinction coefficient (22400 M^{-1} cm^{-1}).

Electron spin resonance spectroscopy Raw pork stored at different time was cut into small pieces (about 1 cm^{-3}), lyophilized, and approximately 0.60 g of meat samples were placed to a cylindrical clear-fused quartz tube (outside diameter, 5 mm; Wilmad Glass Company Inc., Buena, NJ, USA). The electron spin resonance (ESR) was measured using an EMX plus ESR spectrometer (Bruker, Rheinstetten, Germany) equipped with an ER 4103 TM cavity. Instrument parameters were set as follows: center field, 3516.75 G; microwave power, 20 mW; sweep width, 200 G; receiver gain, 30 dB; modulation amplitude, 5.000 G; total scan time, 40 s. WINEPR version 921201 software (Bruker) was used to analyze the spectra and ESR signals are expressed using peak height.

Sample preparation for LC-MS/MS Sample preparation was based on the reported methods (24) with some modifications. A quantity of sample (equivalent to 5 mg protein) was mixed with 2 mL n-hexane and centrifuged for 15 min (4,000× *g*). The upper procedures were repeated three times to completely remove the lipid. Then, the sample was reduced with 1.5 mL sodium borate buffer (0.2 M, pH 9.4) and 1 mL sodium borohydride (1 M in 0.1 M sodium hydroxide) at 4°C for 8 h, and then 2.5 mL concentrated HCl (12 M) was added to the above solution. The mixture was incubated at 110°C for 24 h for hydrolysis. The hydrolysate was filtered and diluted to 10 mL. Two hundred microliters of diluted acid hydrolysate were evaporated under nitrogen gas at 50°C and reconstituted with 150 μL of 0.3 μg/mL d4-CML and 150 μL of 0.3 μg/mL d4-CEL in 2 mL of aqueous 5 mM NFPA. The reconstituted acid hydrolytes were vortex mixed for 0.5 min and subjected to SPE. For the SPE, an Oasis MCX cartridge (60 mg, 3 cc, 60 μm) was preconditioned using 3 mL methanol and 3 mL of 5 mM NFPA. The hydrolysate was loaded on cartridge, and then washed with 3 mL of 5 mM NFPA and 3 mL methanol. The retained CML, CEL, d_4-CML, and d_4-CEL were finally eluted by 5 mL of eluent (methanol/ammonium hydroxide, 95/5, v/v). The eluate was evaporated under nitrogen gas at 45°C and dissolved in 200 μL of 5 mM NFPA in ultrapure water, vortex mixed for 0.5 min, and then filtered through a 0.22-μm fiber membrane prior to analysis by HPLC-MS/MS.

LC-MS/MS analysis The HPLC separation was performed using a Waters 2695 separations module, coupled with a Waters XBridge™ C_{18} column (2.1 × 100 mm, 3.5 μm) housed in a column oven at 35°C, using acetonitrile as solvent A and 5 mM NFPA aqueous solution as solvent B. The flow rate was set at 0.3 mL/min and the injection volume was 5 μL. The gradient was employed starting at 5% solvent A, which was maintained for 0.1 min, and followed by a linear gradient from 5 to 60% solvent A in 4.9 min, then a faster linear gradient from 60 to 100% solvent A in 2 min, with a hold at 100% solvent for 2 min, and return to 5% solvent A for 1 min. MS was operated in positive electrospray ionization (ESI+) mode with multiple reaction monitoring. Typical tuning parameters were cone voltage, 20 V; capillary voltage, 3.55 kV; desolvation temperature, 350°C; source temperature, 110°C; desolvation gas (nitrogen, 99.9% purity) flow rate, 450 L/h; cone gas flow rate, 50 L/h; collision gas (argon, 99.9999% purity) flow rate, 0.15 mL/min. Mass spectrometric parameters are shown in Table 1. CML and CEL were quantified based on isotopically labelled internal standards and calculated according to an external standard calibration curve in all samples.

Table 1. Mass spectrometric parameters for multiple reactions monitoring AGEs

Compounds	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (ev)
CML	205	84	24	14
D_4-CML	209	88	26	15
CEL	219	84	28	16
D_4-CEL	223	88	26	13

Statistical analysis All the experiments were run in triplicate and the LC-MS/MS experiments were repeated twice. Results are expressed as mean±standard deviation. Statistical analyses were performed using SPSS statistical program (version 20.0 for Windows, SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used to analyze data, and Duncan's multiple-range tests were conducted to determine differences between the means. Statistical significance for all analyses was accepted at the level of $p<0.05$.

Results and Discussion

Performance of the analytical method The methods used in this study were verified by calibration, sensitivity, and a recovery test. The correlation coefficients (R^2) of the CML and CEL calibration curves were 0.9977 and 0.9998, respectively. The linear ranges of CML (30.97-4220 ng/mL) and CEL (33.59-4300 ng/mL) were wide enough in all samples. Limits of detection were 8.2 and 9.4 ng/mL, respectively. Limits of quantification were 27.4 and 30.7 ng/mL, respectively. CML and CEL recoveries were 90.33-103.15% and 90.45-95.18%, respectively. These results show that this method is suitable for determining the CML and CEL contents in our study.

Changes in TBARs, carbonyl content, and ESR in stored raw pork The TBARs value has been commonly used as an oxidation index of lipids in foods (25). In order to investigate the extent of lipid oxidation during storage of raw pork, TBARs was determined. As shown in Fig. 2A, there was a significant increase in TBARs value in raw pork stored at 18°C for 3 months, while no significant difference in TBARs value was observed after 3 months of storage, which suggested that lipid oxidation in frozen raw meats mainly occurred in early stages of storage (3 months). This finding was in accordance with a previous study. Brewer *et al.* (26) suggested that the effect of packaging on lipid oxidation in ground pork, and the result indicated that TBARs values in vacuum package (0.65 mg MDA/kg sample) and polyvinyl chloride film package (1.30 mg MDA/kg sample) were markedly higher than that in fresh pork (0.20 mg MDA/kg sample) after long-term frozen storage. Vieira *et al.* (27) also stated that the TBARs value of fresh meat was significantly lower than that meat stored for 90 days at 20°C.

Protein carbonyl has been widely applied to the assessment of protein oxidation (28). As shown in Fig. 2B, carbonyl content of fresh pork was 1.35 nmol/mg, and there was a significant increase in carbonyl content with increasing storage time. After 3 months of storage, the highest carbonyl content was observed, suggesting that protein oxidation was considerable in frozen pork after 3 months of storage. Protein oxidation is believed to proceed via a free radical chain reaction similar to that of lipid oxidation (29). Estévez (30) also reported that lipid-derived reactive oxygen species (such as peroxyl radicals) are potential initiators of protein oxidation. Therefore, lipid oxidation may contribute to inducing the oxidation of protein during storage of raw pork.

Free radicals are important intermediates formed during lipid oxidation and are involved in the transformation of primary to secondary lipid oxidation products. To investigate the free radical contents during storage of raw pork, ESR spectroscopy was applied. As shown in Fig. 2C, the free radical content in frozen raw pork increased with storage time, which suggested that free radical would be formed slowly during storage.

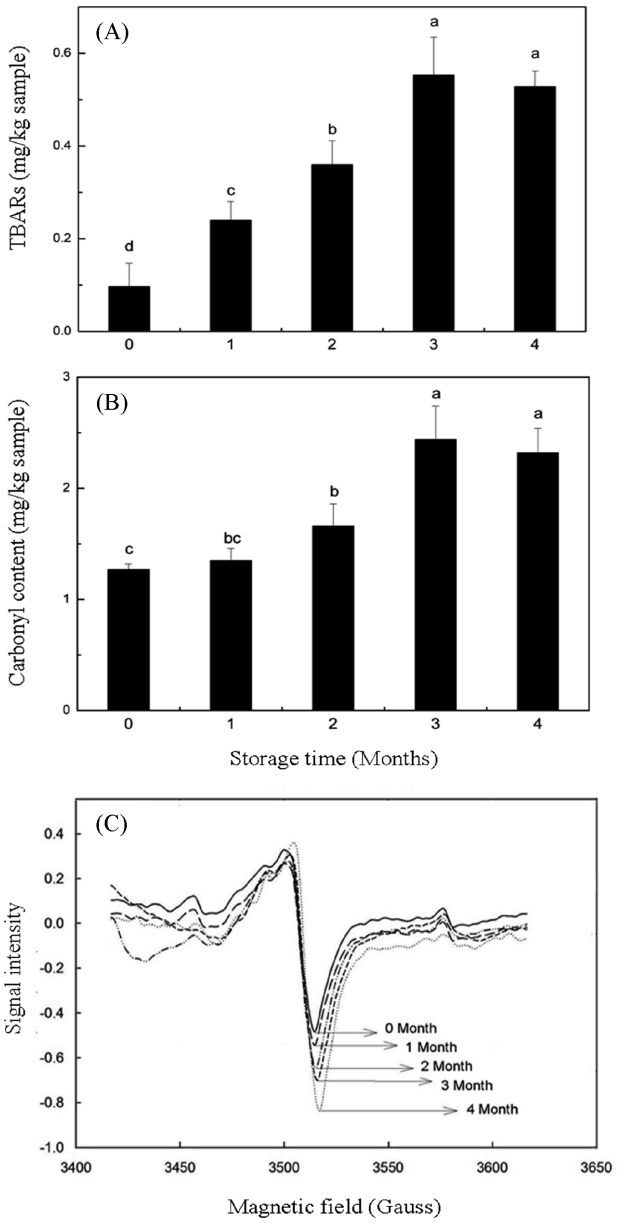

Fig. 2. TBARs (A), carbonyl content (B), and ESR (C) in different storage times of raw pork. Letters a-d above the columns indicate significant differences (p<0.05).

Effect of the sterilization method on CML and CEL formation To illustrate the effects of different heat process methods on CML and CEL formation, the sterilized and pasteurized meat products were made from fresh raw pork, and CML and CEL contents were determined. As shown in Table 2, the contents of CML and CEL in fresh raw pork were 32.52±10.08 and 10.70±2.10 mg/kg protein, respectively. After processing, CML contents in sterilized and pasteurized meat products were 2- and 3-fold higher than that in fresh pork, respectively, and CEL contents in sterilized and pasteurized meat products were 10- and 4-fold higher than that in fresh pork,

Table 2. CML and CEL contents in fresh raw pork and sterilized and pasteurized meat products[1)]

Sample	CML (mg/kg protein)	CML (mg/100 g food)	CEL (mg/kg protein)	CEL (mg/100 g food)
Raw pork	32.52±10.08$^{c1)}$	0.62±0.19^{c}	10.70±2.10^{c}	0.23±0.04^{c}
Sterilized meat products[2)]	64.36±9.11^{b}	1.84±0.26^{b}	114.29±7.35^{a}	3.27±0.21^{a}
Pasteurized meat products[2)]	103.98±2.90^{a}	2.23±0.06^{a}	47.29±9.78^{b}	0.99±0.17^{b}

[1)]Letters a-c in the columns indicate significant differences (p<0.05).
[2)]Meat products were from fresh raw pork (0 weeks of storage).

respectively. These results suggested that processing of meat products could significantly accelerate the generation of CML and CEL.

The content of CEL in sterilized meat products was higher than that of CML, most probably due to the reaction rate of MGO with lysine occurring at a faster rate than the reaction of GO with lysine at high processing temperature (31). Alternatively, the yield of MGO might be higher than GO (32) during a high-temperature condition, resulting in the high content of CEL. Srey *et al.* (31) also reported that the content of CEL in sponge cake prepared at 190°C (30 min) was higher than that of CML. This result was in accordance with our finding.

In addition, we found that the CML content in pasteurized meat products (103.98±2.90 mg/kg protein) was 1.62-fold higher than that in sterilized meat products (64.36±9.11 mg/kg protein), while the CEL content in pasteurized meat products (47.29±9.78 mg/kg protein) was 2.42-fold lower than that in sterilized meat products (114.29± 7.35 mg/kg protein). These results indicated that mild heating temperature (85°C, 4 h) seemed to favor CML formation, while high processing temperature (121°C, 30 min) could promote the generation of CEL. Ahmed *et al*. (33) reported that the content of CML (877±47 nM) was lower than that of CEL (1809±337 nM) in pasteurized milk, while it was higher (2066±497 nM) than that of CEL (1537±104 nM) in sterilized milk. Srey *et al.* (31) also suggested that the reaction rate of MGO (precursor of CEL) with lysine might be different from the reaction rate of GO (precursor of CML) with lysine under different processing conditions. Therefore, the formation pathway or generation mechanism of CML and CEL might differ in sterilized and pasteurized meat products. Further study is needed to investigate the formation kinetics of CML and CEL in different processing temperatures.

Effects of stored raw pork on CML and CEL formation Raw pork was stored at 18°C for 0, 1, 2, 3, and 4 months and were made into sterilized and pasteurized meat products. Contents of CML and CEL were measured in raw pork as well as in sterilized and pasteurized meat products.

As shown in Fig. 3, there was a marked increase in CML and CEL contents in the sterilized meat products made using different storage times of raw pork, and the highest CML and CEL contents were observed in sterilized meat products made using raw pork stored for 4 months (Fig. 3). In addition, a linear correlation was found between TBARs value and either CML (r^2 =0.881-0.898, p<0.01) or CEL (r^2 = 0.958-0.960, p<0.01), carbonyl content and either CML (r^2 =0.961-0.973, p<0.01) or CEL (r^2 =0.967-0.972, p<0.01) in the sterilized meat products made from raw pork with different storage times (Table 3), which suggested that lipid and protein oxidation may promote the formation of CML and CEL in high processing temperature (121°C, 30 min). Lipid and protein oxidation of raw pork will inevitably occur during storage and processing (Fig. 2). These oxidative changes (such as cleavage of peptide bonds and exposure of reactive amino acid residues) (34) may contribute to the generation of CML and CEL via

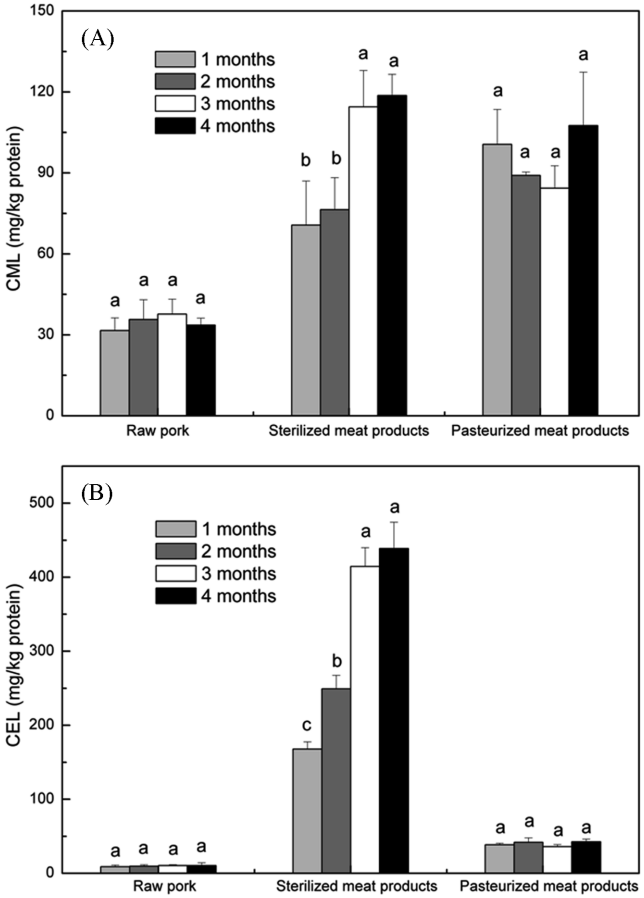

Fig. 3. CML (A) and CEL (B) contents in sterilized and pasteurized meat products made from stored raw pork (1-4 months). Letters a-c above the columns indicate significant differences (p<0.05).

Table 3. Relationship between TBARs (mg/kg sample) or carbonyl content and either CML or CEL in sterilized meat products made from raw pork with different storage times

	TBARs	Carbonyl content
CML (mg/100 g food)	r^2=0.898	r^2=0.973
CML (mg/kg protein)	r^2=0.881	r^2=0.961
CEL (mg/100 g food)	r^2=0.958	r^2=0.967
CEL (mg/kg protein)	r^2=0.960	r^2=0.972

the Maillard reaction pathway during high temperature processing (121°C, 30 min). Alternatively, CML and CEL were also formed through the lipid oxidation pathway (35), and the reactions between the lysine residues and dicarbonyls (such as GO and MGO) formed during lipid oxidation would lead to CML and CEL formation. This reaction pathway may be conducive to the generation of CML and CEL during high temperature processing.

In pasteurized meat products (85°C, 4 h), no significant difference was found in CML and CEL contents regardless of the raw pork storage time. These data indicated that lipid and protein oxidation may not significantly affect CML and CEL generation under mild processing temperature (85°C, 4 h).

In conclusion, the effects of stored raw meat and process procedures

on CML and CEL generation were investigated in the present work. Under high processing temperature (121°C, 30 min), the stored raw pork could promote the formation of CML and CEL, while stored raw pork did not affect the CML and CEL generation in mild processing conditions (85°C, 4 h). Compared with fresh raw meat, thermal processing could accelerate CML and CEL generation. Mild heating temperatures (85°C, 4 h) seemed to favor the generation of CML, while high processing temperatures (121°C, 30 min) could significantly promote the formation of CEL. Therefore, the formation mechanism of CML and CEL might be different in different processing conditions. Further study is needed to investigate the kinetics of CML and CEL formation under different processing temperatures.

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Disclosure The authors declare no conflict of interest.

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